

Diagnostic-test evaluation of immunoassays for anti-*Toxoplasma gondii* IgG antibodies in a random sample of Mexican population

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Abstract

Introduction: There are few articles on evaluation of *Toxoplasma gondii* serological tests. Besides, commercially available tests are not always useful and are expensive for studies in open population. The aim of this study was to evaluate in-house ELISA and western blot for IgG antibodies in a representative sample of people living in Mexico.

Methodology: Three hundred and five serum samples were randomly selected from two national seroepidemiological survey banks; they were taken from men and women of all ages and from all areas of the country. ELISA cut-off was established using the mean plus three standard deviations of negative samples. Western blots were analysed by two experienced technicians and positivity was established according to the presence of at least three diagnostic bands. A commercial ELISA kit was used as a third test. Two reference standards were built up: one using concordant results of two assays leaving the evaluated test out and the other in which the evaluated test was included (IN) with at least two concordant results to define diagnosis.

Results: the lowest values of diagnostic parameters were obtained with the OUT reference standards: in-house ELISA had 96.9% sensitivity, 62.1% specificity, 49.6% PPV, 98.1% NPV and 71.8% accuracy, while western blot presented 81.8%, 89.7%, 84.0%, 88.2% and 86.6% values and the best kappa coefficient (0.72-0.82).

Conclusions: The in-house ELISA is useful for screening people of Mexico, due to its high sensitivity, while western blot may be used to confirm diagnosis. These techniques might prove useful in other Latin American countries.

Key words: ELISA; Mexico; *Toxoplasma gondii*; assay validation; western blot

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Introduction

The protozoan parasite *Toxoplasma gondii* was first described more than a century ago. It is the cause of toxoplasmosis, a zoonotic disease that infects many warm-blooded animals, including humans, but with variable frequency among regions [1,2]. Natural infection is acquired by ingestion of tissue cysts or oocysts present in food or water. The immunity developed during primo-infection generally protects the host against new challenges, but the parasite persists in the tissues lifelong [3,4]. Neonatal disease, stillbirth and abortion can be the result of a primary infection occurred during pregnancy in humans and animals [5]. Besides, *T. gondii* is among the opportunistic pathogens in AIDS patients, causing severe encephalitis [6].

Different serological tests have been used for *T. gondii* infection diagnosis, such as the Sabin-Feldman (dye) test, indirect fluorescence (IFAT), ELISA and western blot [7]. Numerous commercial enzyme immunoassays are currently available to detect anti-*T. gondii* antibodies [8,9]. However, they are often costly, especially for screening or epidemiological studies. An alternative is to develop and validate in-house immunoassays. Since the outcome of epidemiological studies is determined by the performance of the diagnostic tests employed, their validation is important to correctly establish prevalence [10].

Several studies about seroprevalence of *T. gondii* in Mexico have been done, but the diagnostic performance of the tests used was not reported [11-13]. A capture-IgM commercial kit and an indirect

IgG-ELISA against the immunodominant surface antigen of *T. gondii* (SAG1) were validated in a small population of pregnant women [14,15]. In addition, we used IgG and IgM western blot to confirm adult and newborn cases [16]. Nevertheless, there are no reports on the validation of ELISA or western blot to detect or confirm cases of *T. gondii* infection in open population. The diagnostic parameters of the tests may also vary among people from different regions, age and gender, since exposure degree and parasite antigens may vary.

In order to carry out the diagnostic-test evaluation, several approaches have been undertaken, although none is perfect [reviewed in 10]. The aim of the present study was to standardise and evaluate an indirect in-house ELISA and a western blot assay for anti-*T. gondii* IgG antibody determination, using human serum banks collected in two National Health Surveys of Mexico.

Methodology

The Research and Ethics Committees of the National Institute of Pediatrics of the Ministry of Health approved the present work (Registration number 025/2009).

Selected populations, sample size and study design

Although there is no consensus in the literature regarding adequate sample size for diagnostic-test validations, a global figure of at least 200 surveyed individuals has been proposed [17,18]. In order to increase the power of the study, three hundred five sera were randomly selected from the banks of the Mexican National Health Survey 2000 (NHS-00) and the Mexican National Health and Nutrition Survey 2006 (NHNS-06). The former was created as part of the System for National Health Surveys (November 1999–June 2000) while the second was designed to determine the nutritional status of the Mexican population in different regions of the country (November 2005–December 2006) [19,20]. Samples from both surveys are kept in the Serum Bank

established at the National Institute of Public Health, Cuernavaca, Morelos state, Mexico. The present study included serum samples from persons of both surveys, the whole country classified in three regions (centre, coast and north) both genders and three age groups: children (1-10 years) adolescents (10–19) and adults (20–39) (Table 1).

Two hundred microliters of each sample were used to detect anti-*T. gondii* IgG antibodies by indirect ELISA and by western blot. These tests were previously standardized and validated in our laboratory for pregnant women, with diagnostic sensitivity (DSe) and specificity (DSp) above 94% and 78%, respectively [14]. Additionally, samples were analysed for *Toxoplasma*-specific IgG antibodies using a commercial available immunoassay.

ELISA

Preliminary standardization of antigen concentration and serum dilution was done with positive and negative controls and reported [14]. Polystyrene plates (Maxisorp Nunc) were coated with 100 µL/well of *T. gondii* RH strain tachyzoites crude extract (2µg/mL) in 0.015 M carbonate buffer, pH 9.6 at 4°C overnight. Each well was blocked with 200 µL of 10 mM phosphate buffered 0.15 M saline (PBS), pH 7.4, containing 1% bovine serum albumin (Euro-Clone, Milano, Italy) and 0.05% Tween 20. Plates were washed 5 times with PBS-Tween (PBS-T) using an automated BIO-RAD 1575 ImmunoWash machine (Bio-Rad Laboratories, Hercules, USA) and incubated with 100 µL of serum diluted 1:500 in PBS-T at 4°C overnight. The plates were washed as indicated above and incubated with 100 µL/well of a goat anti-human IgG-peroxidase conjugate (Sigma-Aldrich Co., St Louis, USA, product A8419) diluted 1:5000 in PBS-T, 2 hours at 37°C. After further washing, the antigen-antibody reaction was developed by addition of 100 µL/well of the substrate/chromogen solution (5 mL of 0.1 M citric acid plus 5 mL of 0.1 M sodium citrate, added with 5 mg O-phenylenediamine [Sigma-Aldrich] and 4.5 µL of 30% H₂O₂). Plates were

Table 1. Human being sera analyzed for anti-*T. gondii* IgG according to age, gender and geographical area of Mexico.

Age	n (%)	Men	Women	Center ¹	Coast ²	North ³
Children (1-9 y)	106 (34.8)	53	53	37	34	35
Adolescents (10-19 y)	91 (29.8)	49	42	32	29	30
Adults (20-39 y)	108 (35.4)	52	56	35	37	36
Total (%)	305	154 (50.5)	151 (49.5)	104 (34.1)	100 (32.8)	101 (33.1)

¹Ten states localized below the Tropic of Cancer but without coast.

²Seven western states with coastline on the Pacific Ocean and five eastern states with coastline on the Gulf of Mexico or the Caribbean Sea.

³Ten states localized above the Tropic of Cancer and at USA-Mexico border.

incubated in the dark at room temperature and the reaction was stopped by addition of 50 μ L/well 1.0 N sulphuric acid. Absorbance was measured at 490 nm on a *Turner Biosystems* 9300-010 ELISA-Modulus Microplate Reader. Cut off was set as the absorbance mean of three *T. gondii* negative samples previously tested (run in duplicate) plus 3 times the standard deviation. In order to establish the Reactivity Index (RI) the absorbance of each tested sample was divided by the cut-off. Serum samples with $RI \geq 1.1$ were considered positive and undetermined samples (RI from 1.0 to 1.1) were classified as negative. RI frequency was plotted on a distribution curve (Figure 1). Four technicians carried out the ELISAs and the intra- and inter-assay variation coefficients were calculated according to standard procedures [21].

Western blot

The method was performed as previously reported [14,16]. Briefly, 1.5×10^7 >95% pure RH strain tachyzoites per gel were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions and transferred onto nitrocellulose membranes (Hybond-C pure, Amersham Pharmacia Biotech, Piscataway, USA) [22,23]. The membranes were blocked with 2% skim milk for 1 hour, washed thrice with PBS-T and incubated with the serum samples diluted 1:200. After further washing, they were developed by incubation with anti-human IgG-peroxidase conjugate (Sigma-Aldrich, St. Louis, USA) diluted 1:2500 in PBS-T. The immune complexes were developed by 60 mg of 4-chloro-1-naphthol (Sigma-Aldrich, St. Louis, USA) in 10 mL methanol added with 10 mL of PBS plus 100 μ L of 30% H_2O_2 . Two experienced diagnosticians independently evaluated antigenic patterns and the frequency of each band. Serum samples were considered positive when at least three of the main antigenic bands (see below) were detected in the nitrocellulose strip.

Commercial Kit

Serum samples were also analysed for anti-*T. gondii* IgG antibodies using a commercial enzyme immunoassay (VIDAS TOXO IgG II, kit Ref 30 210, BioMérieux sa, 69280 Marcy-I'Etoile, France) following the manufacturer instructions and reading the absorbance at 450 nm with a *mini VIDAS* ELISA auto-reader.

Validation

After serologic techniques were carried out, three results were obtained for each sample (ELISA,

western blot and VIDAS). Evaluation of both in-house immunoassays was carried out in two ways: in the first model (“IN”) the test to be validated was included to construct a reference standard, which result was determined on the basis of at least two concordant (positive or negative) results. In the second approach (“OUT”) the reference was built up with the results of the non-evaluated tests, *i.e.* in-house ELISA was contrasted with western blot and VIDAS concordant results and western blot with in-house ELISA and VIDAS. Diagnostic parameters were DSe, DS_p, positive and negative predictive values (PPV and NPV), positive and negative likelihood ratios and kappa, with 95% confidence intervals [24]. Data were stratified by gender, age and region, stored in a database created in Excel 2007 and processed for analysis. The Rogan-Gladen (RG) estimator was used to calculate “true” prevalence of *T. gondii* infection:

$$RG = \frac{AP + DS_p - 1}{DSe + DS_p - 1}$$

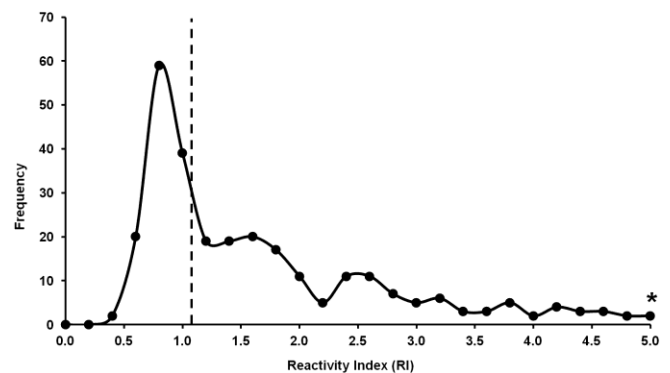
Where: AP = Apparent prevalence; DS_p = Specificity; DSe = Sensibility [25].

Results

Figure 1 shows the reactivity index distribution obtained by ELISA. A group of possible negative samples ($RI \leq 1.1$) can be seen at the left of the plot; they were later confirmed negative in the other two assays. Intra assay, inter assay and inter technician variation coefficients were 1%, 5% and 11%, respectively.

Heterogeneous 14-113 kDa band patterns were obtained in western blot, with an antigen of around 35 kDa being the strongest and more frequently recognized, followed by three bands larger than 78

Figure 1. Frequency distribution of absorbance values obtained by ELISA on 305 human serum samples. Dashed line: cut-off point ($RI = 1.1$). *Twenty-seven cases had $RI \geq 5.0 \leq 11.0$.



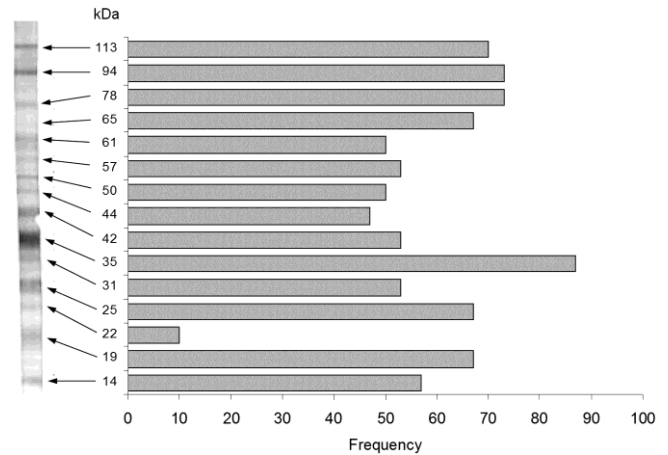
kDa (Figure 2). Then, all 305 samples of the National Banks were tested and used in western blot validation. Agreement between diagnosticians was 92% and concordance between ELISA and western blot was 0.69 and 0.70 for each technician.

The diagnostic performance values of immunoassays are shown in Table 2. ELISA presented the highest DSe (98.4% CI₉₅ = 94.2-99.5 and 96.9%, CI₉₅ = 89.5-99.2 IN and OUT respectively) and NPVs (98.3%, CI₉₅ = 94.2-99.5 and 98.1%, CI₉₅ = 93.4-99.5) as well as accuracies above 71%. Globally, western blot was the best assay, with good kappa coefficient for both evaluations (κ = 0.72 and 0.82) and up to 91.5% diagnostic accuracy (CI₉₅ = 87.8-94.1).

In house-ELISA DSe and DSp did not significantly vary among gender, age or region groups. ELISA OUT lowest DSe was 94.1% (CI₉₅=73.0-99.0) among children and 94.4% (CI₉₅=74.2-99.0%) among people living in the centre of the country. DSp was not higher than 73.3% and 75.8% for ELISA OUT and IN, respectively. Globally, WB OUT showed the best DSp independently on the group, being the lowest 86.4% in both women (CI₉₅ = 75.5-93.0) and children (CI₉₅ = 73.3-93.6) and up to 93.1% in men (CI₉₅ = 83.6-97.3).

Recently, we reported the seroprevalence of

Figure 2. Typical band pattern observed with positive human serum samples by western blot (strip) and frequency of each band recognized by IgG anti-*T. gondii* antibodies, according to its molecular weight. Evaluation was performed with one hundred samples.



human toxoplasmosis in Mexico using the ELISA IN sensitivity and specificity [26]. In the present study adjusted prevalence by Rogan-Gladen estimator was recalculated with the ELISA OUT and no significant variations were observed neither in the 2000 nor in the 2006 surveys (Table 3) [25].

Table 2. Diagnostic values of ELISA, Western blot and commercial available assay for detection of IgG anti-*T. gondii* infection in human serum samples.

	Reference Standard ¹					
	ELISA OUT ² (n=234)	ELISA IN (n=305)	WB OUT ² (n=194)	WB IN (n=305)	VIDAS OUT ² (n=213)	VIDAS IN (n=305)
Diagnostic Sensitivity (DSe)	96.9	98.4	81.8	87.8	59.4	64.8
Diagnostic Specificity (DSp)	62.1	65.0	89.7	94.0	98.1	98.9
Positive Predictive Value (PPV)	49.6	65.2	84.0	90.0	96.9	97.5
Negative Predictive Value (NPV)	98.1	98.3	88.2	92.4	70.9	80.8
Kappa	0.46	0.58	0.72	0.82	0.58	0.67
Likelihood ratio positive	2.6	2.8	8.0	13.5	31.8	59.3
Likelihood ratio negative	0.05	0.03	0.20	0.12	0.41	0.36
Proportion positive false	37.9	35.0	10.3	6.6	1.9	1.1
Proportion negative false	3.1	1.6	18.2	11.5	40.6	35.2
Diagnostic accuracy	71.8	78.4	86.6	91.5	78.9	85.2

¹Evaluated test was considered **OUT** (agreement between two remaining immunoassays) or **IN** (agreement of at least two of three test results) of the Reference Standard. WB: Western blot. VIDAS Toxo IgG II.

²Number of samples in OUT validations were lower since those with non-concordant results of the other two tests were excluded.

Table 3. Changes in ELISA sensitivity and specificity when it is included (**IN**) or not (**OUT**) in the Reference Standard and influence on adjusted prevalence of two Mexican National Health Surveys

Survey	Parameter	ELISA IN*	ELISA OUT	Adjusted prevalence	Adjusted prevalence
		Reference Standard (%)	Reference Standard (%)	ELISA IN* (%)	ELISA OUT (%)
National Health Survey-2000	Sensitivity	98.4	96.9	40.0	37.6
	Specificity	65.4	62.1		
National Health Nutrition Survey-2006	Sensitivity	98.4	96.9	43.1	41.9
	Specificity	65.4	62.1		

* Diagnostic values and adjusted (IN) prevalence were previously reported for each survey (Caballero-Ortega *et al.*, 2012). Adjusted prevalence was calculated by the Rogan-Gladen estimator (Rogan and Gladen, 1978).

Discussion

Since *Toxoplasma gondii* causes a life-lasting infection with persistent antibodies, serological tests are useful tools to diagnose it at individual and population levels [3,4]. Thus, it is critical to have access to rapid and sensitive methods in order to support screening risk groups (like pregnant women, HIV or otherwise immunosuppressed patients) and for epidemiological studies. Despite the need for known diagnostic parameters of a test in order to correctly interpret epidemiological or screening results, few evaluations have been published recently [10]. Validations of *T. gondii* IgG-antibody detection tests are scarce worldwide and have been mainly done with few samples, not necessarily representative of the population [9,14,15,27].

Mexico has done a huge effort to generate nationwide information about human diseases prevalence and some risk factors, by means of national surveys, some of which have included serum sample banks. The National Seroepidemiological Survey performed in 1987 was used for several diseases, among them toxoplasmosis, which was studied analysing IgG antibodies by IFAT [11]. The diagnostic parameters of the assay were not reported in that study, but a 90% concordance with a Canadian reference laboratory is mentioned; thus, prevalence was not calculated taking into account diagnostic performance of the test. As it is shown in the present study of the 2000 and 2006 serosurveys, ELISA was satisfactory in terms of sensitivity, regardless of gender, age or region. It is also cheap (less than a dollar per sample) and easy to perform. Moreover, the variation coefficients among four technicians were within good diagnostic performance. It presented a low PPV, mainly in low frequency groups (children) or regions (northern Mexico) but this was expected due to the known effect of prevalence on this parameter; adjusting the theoretical frequency to 50% it rendered a PPV close to 70% (data not shown). Likelihood ratio was within adequate intervals, considering that the study was performed with samples from open population; therefore, it is useful as a screening test [28,29].

The western blot, as read by either diagnostician, showed satisfactory DSp than DSe and presented the highest global accuracy. Even in high frequency groups (adults or people living on the coast) the PPN was adequate –above 88%. Nevertheless, its use in field or screening studies is precluded due to its high cost and time consumption. One important finding was that diagnosticians presented high agreement between

them; also, a good concordance between ELISA and western blot was obtained, which makes the latter technique suitable for confirmation of cases. As a matter of fact it has been proposed for these purposes before [9].

Conclusion

The IgG ELISA designed for this study is useful as a screening test for serum samples of male and female Mexican people from any area of the country, while western blot is suggested as confirmatory test. Due to geographical and race similarities, these techniques could be useful in other Latin American countries.

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